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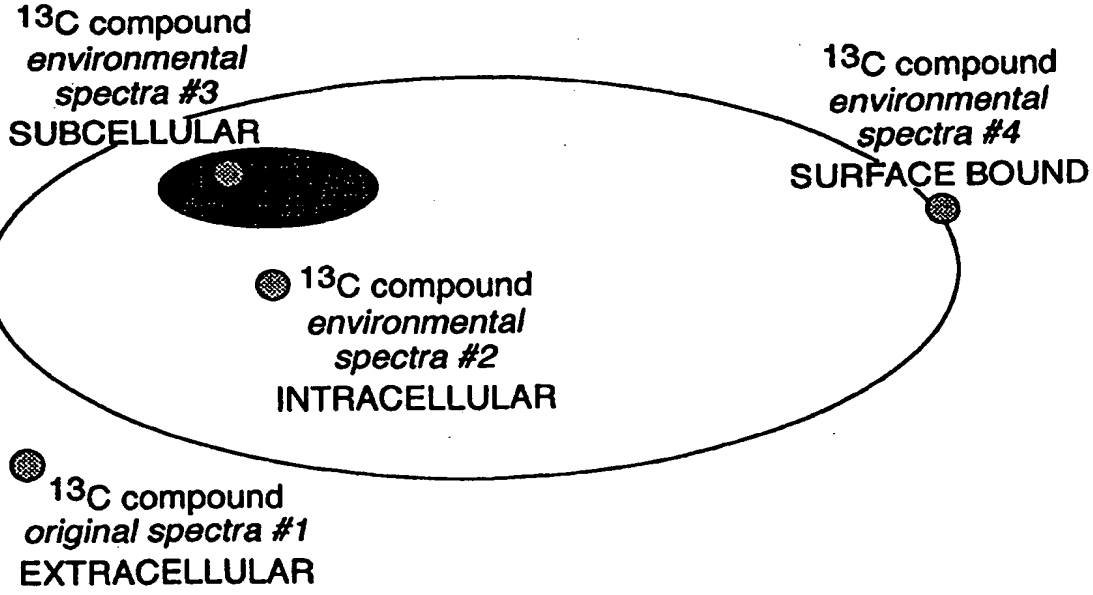
(54) Title: NMR SPECTROSCOPIC IN VITRO ASSAY USING HYPERPOLARIZATION



(57) Abstract

An *in vitro* assay method which comprises the use of an assay reagent containing at least one NMR active nucleus, and hyperpolarising at least one NMR active nucleus of the assay reagent; and analysing the assay reagent and/or the assay by NMR spectroscopy and/or NMR imaging. The assay reagent may contain an artificially high concentration of an NMR active nucleus.

NMR SPECTROSCOPIC IN VITRO ASSAY USING HYPERPOLARIZATION

TECHNICAL FIELD

The present invention relates to tailoring the shape of a magneto-resistive material, and more particularly to a design of the shape of the magneto-resistive material to obtain a new type of position sensitive sensor.

BACKGROUND

The position of a moving object is often determined by means of the readout from a resistive sensor, usually of potentiometer type, which is mechanically connected to the object to be monitored.

In order to reduce the wear and thereby increase the reliability, it is desirable to eliminate the sliding friction encountered in the standard resistive sensors. Non-contact methods using e.g. inductively coupled coils is currently being introduced as replacement for the potentiometer sensors. However, these are more complex and therefore more expensive.

In recent years novel types of magneto-resistive materials with much higher sensitivity to moderate changes in magnetic fields have been found. These new materials showing giant magneto-resistance (GMR) or colossal magneto-resistance (CMR) make possible new types of position sensors.

In a document U.S. Patent No. 5,475,304 is disclosed a giant magneto-resistant sensor including at least one layered structure. The layered structure includes a ferromagnetic layer having a fixed magnetic state, a second, softer magnetic layer, and a metal layer interposed between and contacting these two layers. The sensor also includes one or more indexing magnets for inducing a domain wall, at a measured position, between regions of nonaligned magnetic fields in the softer magnetic layer. By measuring the resistance across the magneto-resistant sensor a displacement of one workpiece, carrying the sensor, will be measured relative to another workpiece carrying an inducing means.

advantage of this invention is that assay reagent containing an NMR active nucleus may in many cases, provide the same information previously provided by corresponding ^{14}C-labelled compounds, whilst being free from the problems associated with radioactive isotopes.

One further advantage according to the present invention is the increased signal-to-noise ratio. Another improvement with the present invention is that the time required to perform the assay is in general much shorter than the previously known methods. These improved parameters/results may be expressed as a "shortening effect", being the improvement of signal-to-noise ratio per unit time, and will be discussed further.

Yet another advantage compared e.g. with assays using fluorescent reagents is that there is no need to add an additional chemical component to the assay reagent to assist detection. There is always a disadvantage with techniques such as the fluorescent methods because the additional chemical component may influence the measurement.

The present invention provides an *in vitro assay* method which comprises:

a) using an assay reagent containing at least one NMR active nucleus to perform an assay, and
b) hyperpolarising at least one NMR active nucleus of the assay reagent;
Wherein steps a) and b) are performed simultaneously or sequentially in either order, and
c) analysing the assay reagent and/or the assay by NMR, and
d) optionally using the NMR data obtained in step c) to generate further assay result(s).

As used herein, NMR active nuclei are those having non-zero nuclear spin and include ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{29}Si, ^{31}P and/or deuterium. Of these, ^{13}C and ^{15}N are preferred and ^{13}C is particularly preferred. Preferably the assay reagent for use in the assay according to this invention comprises an artificially-enriched abundance of an NMR active nucleus.

In a further preferred embodiment of the invention, the enriched compound comprises the artificially enriched NMR active nuclei, e.g. ^{13}C, at one specific position. Alternatively, in another preferred embodiment the compound comprises enriched NMR active

important aspect of the present invention is thus an assay wherein the time required to give a defined signal-to-noise is considerably shortened by the use of this hyperpolarisation technique compared to known assay techniques without hyperpolarisation. The shortening effect is expressed as the improvement of signal-to-noise ratio per unit time, dB $\sqrt{}$ Hz. This effect is preferably a factor of 10 or more, more preferably a factor of 25 or more and even more preferably a factor of 50 or more. In some embodiments, this effect is particularly a factor of 200 or more or even a factor of 1000 or more.

The assay can be carried out with the NMR active nucleus in the assay reagent already hyperpolarised. Alternatively, the assay may be carried out and the NMR active nucleus subsequently hyperpolarised prior, or at the same time, as the assay/assay reagent is analysed by NMR spectroscopy. Whilst the first arrangement enables real time studies of the assay to be carried out, this is often not necessary and, in these circumstances, the second method is very useful. As hyperpolarisation of the NMR active nucleus will sometimes be carried out at a low temperature, e.g. 20 K or less, the assay can be started and then effectively frozen by lowering the temperature. The assay/assay reagent is then hyperpolarised and analysed by NMR spectroscopy. By carrying out this process a number of times, either on the same assay or on parallel assays, a series of "snap-shots" of how the assay is proceeding may be obtained.

When hyperpolarisation is effected by exchange in solution phase, the hyperpolarising agent can be introduced as one batch, continuously or intermittently. Some conditions would lead to rapid disappearance of the hyperpolarisation. However, continuous or intermittent hyperpolarisation will give adequate signal intensity. Repeating the hyperpolarisation – acquisition sequence will also enhance the signal to noise ratio.

Agents, such as organic solvents, may in some situations be added to the assay, and/or to the NMR active nucleus if this is to be hyperpolarised prior to the assay, in order to prolong the life time of the hyperpolarised NMR active nucleus in the assay reagent, without interfering with the assay reagent and/or assay method.

Assays can be carried out by quantifying the appearance, or the continued presence, or the disappearance of spectral patterns. For example, on binding or hybridisation of an assay

For achieving as long T_1 as possible, the enriched compounds in some methods covered by the invention are preferably those in which the NMR active nucleus is surrounded by a double bond or one or more non-MR active nuclei such as O, S and/or C. In some cases, nearby protons to the NMR active nucleus may be substituted by deuterium.

In one embodiment of the invention, step c) is performed by examining the assay reagent using both NMR spectroscopy to obtain spectral data from one or more discrete physical locations and repeating the examination at least once so as to obtain quantitative information about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.

Assays envisaged according to this invention include for example, competition assays (e.g. receptor-ligand antagonism, enzyme-substrate inhibitors, protein-protein interaction inhibitors), binding assays (e.g. receptor-ligand agonism, enzyme-substrate reactions, protein-protein interactions), immunoassays (e.g. for specific analytes), hybridisation assays (e.g. nuclease assays, mutation analysis, mRNA and DNA detection), tests involving cells, organs and/or whole organisms. Thus, the invention covers binding studies performed on tissue sections, cultured cells, cellular metabolites, micro-organisms and macro-organisms. Preferred examples are discussed in the following paragraphs. Labelling with an NMR active nucleus where each molecule may be labelled at one or more chemical positions, will allow unique NMR assignments of e.g. starting material, intermediates and products of a biological reaction. Thus dual, triple etc labelling experiments can be carried out and 'stop-flow' measurements made with identical chemical species. For example, theoretically, all the six carbon atoms in glucose could be individually or collectively replaced by ^{13}C, so that one to six of the carbon atoms are ^{13}C which can be hyperpolarised. Each hyperpolarised ^{13}C will give rise to a chemical shift, which will be specific to that individual carbon and different to other ^{13}C positions in the molecule, i.e. C-1 will be different from C-2, etc.

In one preferred embodiment of the invention the hyperpolarisation transfer is achieved by using a hyperpolarised noble gas, or a mixture of such gases, to effect nuclear polarisation of an assay reagent comprising at least one NMR active nucleus other than the noble gas.

When the hyperpolarisation of the assay reagent is achieved by an artificially enriched hyperpolarised noble gas, the hyperpolarised noble gas is preferably ^{3}He or ^{129}Xe. Such isotopically enriched gases are now commercially available at high isotope purity and can be polarised to a high degree of hyperpolarisation. The hyperpolarised gas may, if desired, be stored for extended periods of time in the polarised state, by keeping the gas at very low temperatures, especially in a frozen form.

A hyperpolarised noble gas may be used in step b) of the present invention to effect nuclear polarisation of an assay reagent comprising at least one NMR active nucleus other than the noble gas. The hyperpolarised gas may be in the gas phase, condensed or may alternatively be liquid e.g. by being dissolved or emulsified in a lipophilic solvent such as a lipid or a fluorocarbon solvent, or in a suspension or a solid e.g. by being adsorbed or frozen on to a solid surface. In some cases, liposomes or microbubbles may encapsulate the hyperpolarised noble gas.

The assay reagent may be solid, semi-solid or fluid. A hyperpolarised gas may be bubbled into a fluid assay system. Alternatively, a hyperpolarised gas solution may be mixed with a fluid assay. The hyperpolarised gas may be cooled and/or maintained in a magnetic field to preserve the hyperpolarisation. Similarly the resulting assay reagent comprising at least one polarised NMR active nucleus may preferably be cooled and/or maintained in a magnetic field in order to preserve the polarisation and/or facilitate polarisation transfer.

One advantage with hyperpolarisation transfer by ^{3}He or ^{129}Xe is that these gases are essentially chemically inert and will not adversely affect the assay reagent or the assay. In addition, as in gaseous form, ^{3}He and/or ^{129}Xe are easily separated from the assay medium, permitting facile repeat studies.

In one embodiment of the invention, when the polarisation transfer occurs in solution, the pressure of xenon is as high as possible, preferably higher than 5 $x10^5$ N/m^2 (5 bar), more preferably higher than 5 x 10^6 N/m^2 (50 bar), even more preferably higher than 1 $x10^7$ N/m^2 (100 bar) and particularly higher than 2 $x10^7$ N/m^2 (200 bar). However, the pressure must never be so high so that the biological molecule will be totally or partly adversely effected.

It is preferred that the solvent comprises as few atoms which possess magnetic moment as possible and is as low magnetogyric ratio as possible. The transfer of polarisation in a highly viscous medium may be followed by solution spectroscopy under high-viscosity conditions (broad lines).

Alternatively, the viscosity may be lowered prior to spectroscopy, either by a change in temperature or by a change in the chemical composition of the solvent. If the high-viscosity medium is formed by a pH-sensitive gel-forming agent, then the viscosity might be lowered e.g. by a change in pH. Changes of temperature, ion-strength as well as the use of specific additives may also be considered.

In a further embodiment, the present invention provides a method wherein the hyperpolarisation transfer is effected by use of a very high field and with very low temperature (Brute force). The magnetic field strength used should be as high as possible, suitably higher than 1T, preferably higher than 5T, more preferably 15T or more and especially preferably 20T or more. The temperature should be very low e.g. 4.2K or less, preferably 1.5K or less, more preferably 1.0K or less, especially preferably 100 mK or less.

US 5479925 discloses a method for generating MR angiograms in which a contrast agent is passed through a small, high field polarising magnet *in vitro* in order to generate a high longitudinal magnetisation in the agent prior to its administration to the subject. However, there is no mention of the use of an enriched NMR active nucleus. When this Brute force method is used, and thermodynamic equilibrium is attained, all nuclei in the assay reagent will be highly polarised relative to room temperature and to normal magnetic fields used in MRI.

Most known paramagnetic compounds may be used as a "DNP agent" in this embodiment of the invention, e.g. transition metals such as chromium ions or organic free radicals such as nitroxide radicals and trityl radicals (WO 98/58272) Where the DNP agent is a paramagnetic free radical, the radical may be convienently prepared *in situ* from a stable radical precursor by a radical-generating step shortly before the polarisation, or alternatively by the use of ionising radiation. Energy, normally in the form of microwave radiation, is provided in the process which will initially excite the paramagnetic species. Upon decay to the ground state, there is a transfer of polarisation to an NMR active nucleus of the target material. The method may be conveniently carried out by using a first magnet for providing the polarising magnetic field and a second magnet for providing the primary field for MR spectroscopy/imaging.

In some cases, the radical will be non-reusable and may conveniently be discarded after use. Many physical and chemical separation or extraction techniques are known in the art, which may be used if it is desirable to remove the DNP agent from the assay system in a rapid and/or efficient separation step. Magnetic properties may e.g. be used to achieve the separation. It is particularly preferred to use a heterogeneous system, e.g. a two-phase liquid, a solid in liquid suspension or a high surface area solid substrate within a liquid. For any heterogeneous system, separation may be achieved by e.g. filtration, decanting, chromatographic or centrifugal methods.

In a further embodiment, the present invention provides a method wherein the polarisation transfer is achieved by exposing the assay reagent to para hydrogen-enriched hydrogen gas in the presence of a suitable catalyst. The assay reagents suitable for use are prepared from precursors which are able to be hydrogenated and which will therefore typically possess one or more unsaturated bonds, e.g. double or triple carbon-carbon bonds.

Hydrogen molecules exist in two different forms, para hydrogen (p-H_2) where the nuclear spins are anti parallel and out of phase (singlet state) and ortho hydrogen (o-H_2) where the spins are parallel or anti parallel and in phase (triplet state). At room temperature, the two forms exist in equilibrium with a 1:3 ratio of para:ortho hydrogen. However, preparation of para hydrogen enriched hydrogen can be carried out at low temperature, 160K or less, in the presence of a catalyst. The para hydrogen formed may be stored for long periods, preferably at low

molecules nearby the NMR active nucleus). The environment thus extends beyond the labelled molecule itself to other molecules in the immediate vicinity. Thus for example, a nucleotide labelled with polarised NMR active nucleus, e.g. ^{13}C and/or ^{15}N, when incorporated into a single stranded polynucleotide chain, can give information about two or more adjacent nucleotide residues in the chain. When that labelled polynucleotide probe is hybridised with a polynucleotide target, NMR spectroscopic analysis of the NMR ^{13}C label can give information about the complementary nucleotide residue in the target.

In one embodiment of the present invention, comparative and/or parallel testing is performed to maximise the information available from the NMR measurements.

Biological macromolecules such as nucleosides or nucleotides or nucleotide analogues can readily be enriched with a NMR active nucleus, e.g. ^{13}C and/or ^{15}N at one or several specified points in the molecule. Polarisation of the NMR active nucleus, e.g. ^{13}C, preferably by contact with a hyperpolarised noble gas, may be effected either before, during or after incorporation of the monomer into a polynucleotide; and before, during or after hybridisation of that polynucleotide with a complementary strand.

Figure 1 demonstrates a hybridisation assay in which the use of an oligonucleotide or polynucleotide is used to detect the presence of single nucleotide polymorphisms (SNPs) in a gene, or fragment of a gene. An oligonucleotide or polynucleotide probe is prepared in which one or more of the atoms has been replaced by a hyperpolarisable isotope, e.g. ^{13}C, ^{15}N or ^{1}H. This probe is then hybridised to the gene or the gene fragment. The probe will be "targeted" to information–rich parts of the gene and may be selected so that the probe binds only to that part of the DNA containing a specific mutation, or, potentially, more than one mutation. If desired, a set of probes, each probe containing a hyperpolarisable isotope, can be added to a gene or gene fragment, each probe being targeted to a different part of the gene/gene fragment. As each probe will have a characteristic chemical shift by NMR spectroscopy, the spectrum of the mixture of the probes with the target can be taken and resolved to indicate which probes have bound and which have not.

The probe may be polarised before, during, or after hybridisation and a determination

The molecule is then brought into contact with an enzyme capable of altering the chemical composition of the substrate. If cleavage occurs between the amino acids containing the hyperpolarisable isotopic atoms, then the J coupling and the chemical shift values change which will be observed by NMR spectoscopy and/or NMR imaging. Two new spectra will appear, one for each of the individual cleavage products. If there is no cleavage, the original spectrum remains.

A similar assay can be carried out where the starting substrate is a chain of nucleotides and the cleavage enzyme an endonuclease.

In another aspect of the invention, an assay reagent may be administered to a macro-organism, e.g. a human or animal, and NMR spectroscopic analysis performed of blood, excreta, e.g. urine, faeces or breath, or samples of the macro-organism.

In yet another aspect of the invention, an assay reagent may be used in binding studies on bacteria or other eukaryotic or prokaryotic micro-organisms or cultured cells.

Assays according to one embodiment of this invention may conveniently be carried out in multiwell plates. An assay reagent in each well may e.g. be hyperpolarised by contact with a hyperpolarised noble gas, prior to addition of other assay reagents. Alternatively, an assay reagent in bulk may be hyperpolarised with a hyperpolarised noble gas prior to being dispensed into individual wells of a multiwell plate. In many cases, assays can be performed in a homogenous mode, that is to say without the need for a separation step to remove one fraction of the labelled reagent.

In addition, in cases where the spectra of the ^{13}C labelled assay components are distinct from one another, more than one assay may be performed and simultaneously monitored in a single well or spot of a multi-assay array. This would allow multiplexing of several related or unrelated assays in parallel within a single well or spot in a multi-assay array which is either ordered or random. In addition the technique may be applied to aerosol droplets where no well, container or surface is used to contain the assay and to analysis of samples in flow-through devices.

A further embodiment of the present invention is an *in vitro* kit for carrying out the assay method as defined. The kit comprises a well, vial or any other suitable container comprising one or more assay reagents optionally together with additives wherein the hyperpolarisation transfer occurs. One embodiment of the invention concerns an *in vitro* kit where the NMR analysis of step (c) of claim 1 is carried out in the same well, vial or container as the polarisation transfer is carried out.

The invention is illustrated with reference to the following non-limiting example. Modifications of the method according to this example include the addition of the noble gas directly into the spectrometer and the use of different pulse techniques.

Example 1.

Polarisation transfer from hyperpolarised ^{129}Xe to the singly labelled peptide AcYRARV(F,^{13}C-amide)FVRAAK-NH_2

Hyperpolarized ^{129}Xe was generated by optical pumping as described by B.Driehuys et al., Appl.Phys.Lett. 69 (12), 1996. The isotopic composition of the gas was 80% ^{129}Xe and 0.25% ^{131}Xe (the rest non-magnetic isotopes of Xe). The degree of polarization was estimated to be10% ±3.

The freeze-dried peptide (3.4 mg) was placed in an ordinary 5 mm thin-walled NMR-tube. The glass tube was connected to the outlet of the polarizer by means of 60 cm of plastic tubing. The tube was evacuated and then filled with nitrogen four times.

The hyperpolarized gas was generated and collected on a cold finger at liquid nitrogen temperature in a holding field of 200 mT over a period of 15 minutes which is estimated to give a volume of 50 ml of Xenon at NTP. A narrow Dewar vessel with liquid nitrogen was placed in a magnet with a field strength of 0.3 T. The collected xenon was thawed and gradually refrozen on the peptide from the bottom and up by gradually lowering the tube into the liquid nitrogen bath. The system was then filled with helium to one atmosphere. The sample, with the plastic tubing still connected but open to the surroundings, in the Dewar in the 0.3 T magnet with the poles in horizontal configuration was then moved into the stray-field of the 7 T magnet (vertical polarity)

CLAIMS

1. An *in vitro assay* method which comprises:

a) using an assay reagent containing at least one NMR active nucleus to perform an assay, and

b) hyperpolarising at least one NMR active nucleus of the assay reagent;

wherein steps (a) and (b) are performed simultaneously or sequentially in either order, and

c) analysing the assay reagent and/or the assay by NMR and

d) optionally using the NMR data obtained in step c) to generate further assay result(s).

2. The method of claim 1 wherein the NMR active nucleus is ^{15}N, ^{19}F, ^{31}P, ^{1}H, ^{29}Si and/or ^{13}C.

3. The method of claim 2, wherein the NMR active nucleus is ^{15}N or ^{13}C.

4. The method of any of claims 1 to 3, wherein the assay reagent is a compound which contains an artificially high concentration of an NMR active nucleus.

5. The method of claim 4, wherein the assay reagent contains an artificially high concentration in 1-10 defined positions.

6. The method of any of claims 1 to 5, wherein the assay reagent is an organic compound comprising one or more NMR active nuclei associated with a bond which is broken during the course of the assay.

7. The method of claim 6, wherein the assay reagent contains two or more NMR active nuclei and each NMR active nucleus produces a distinct NMR spectrum and when the

16. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer from a hyperpolarised noble gas, or a mixture of hyperpolarised noble gases.

17. The method of claim 16 wherein the noble gas is ^{129}Xe.

18. The method of claim 16 wherein the noble gas is ^{3}He.

19. The method of claims 16 to 18 wherein the hyperpolarisation is transferred by a hyperpolarised noble gas in solution and wherein the viscosity of the solution is at least 1000 mPs.

20. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer at a temperature of 4.2 K or less in the presence of a magnetic field of at least 1 T.

21. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer using dynamic nuclear polarisation.

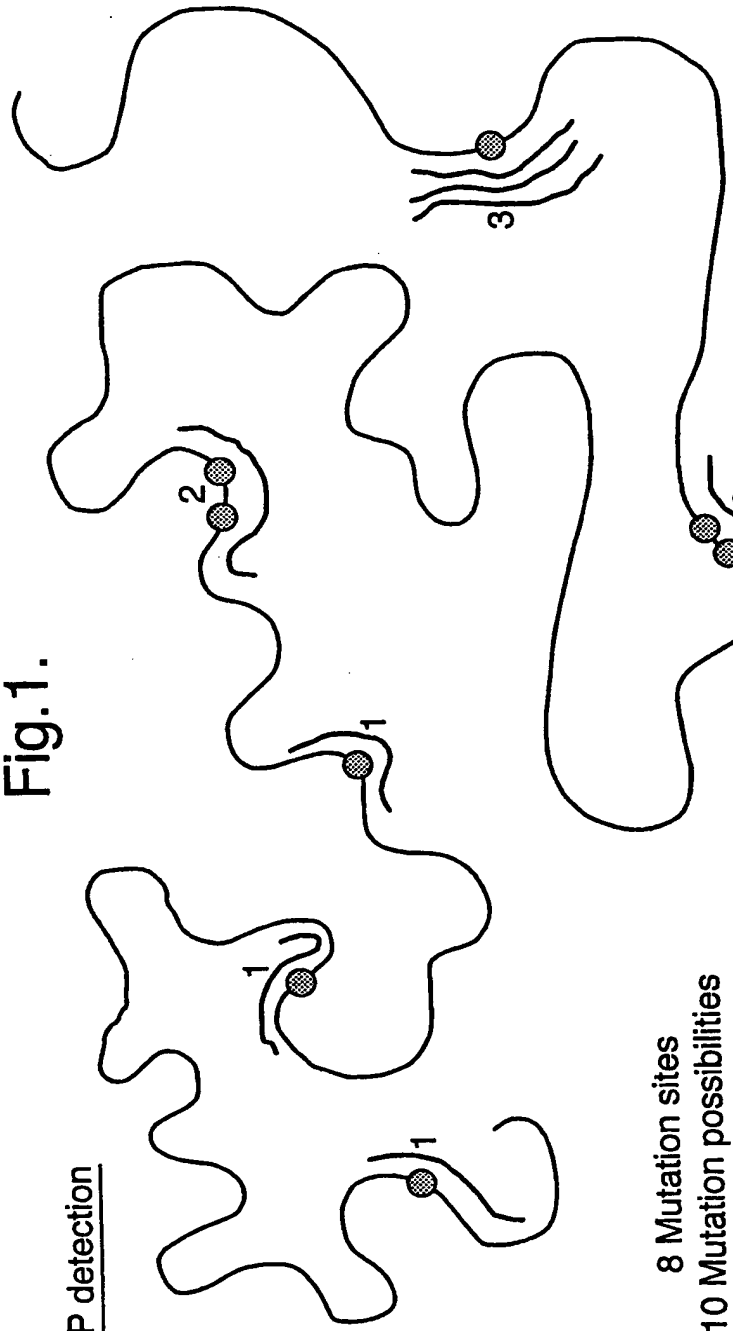
22. The method of claims 1 to15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by para hydrogen induced polarisation.

23. The method of claims 1 to15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out with the spin refrigeration technique.

24. The method of claims 1 to 23, wherein more than one assay is multiplexed and monitored by NMR spectroscopy and/or NMR imaging.

25. The method of claims 1 to 24 wherein the assay is performed in a multiwell or multispot assay array.

Fig.1.


Multiple SNP detection
1
1
1
2
2
3
8 Mutation sites
10 Mutation possibilities
20 Spectra to resolve
1 Simple Spectral Resolution
2 Spin Dipole Spectra
3 Complex Spectral Resolution

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/04410

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01R33/465 G01N33/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01R G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 37239 A (NAVON GIL ;BIFONE ANGELO (IT); ROOM TOOMAS (US); APPELT STEPHAN (U) 9 October 1997 (1997-10-09) cited in the application page 9, line 21 -page 10, line 27 page 14, line 5 -page 19, line 27 page 22, line 23 -page 23, line 38; examples 2-4,7-9	1,8
A	--- -/--	2-7,9-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2000	14/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lersch, W

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/04410

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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